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PRECOCIOUS BREAKDOWN OF THE GERMINAL VESICLE INDUCES PARTHENOGENETIC DEVELOPMENT IN SEA CUCUMBERS*

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Abstract

Meiotic divisions of sea cucumber oocytes can be induced and completed by dithiothreitol (DTT) treatment, but DTT-matured, unfertilized eggs are arrested at the pronucleus stage and do not develop. By pipetting the oocyte suspension, the membrane of the germinal vesicle of the full-grown oocyte ruptured and then disappeared. In immature oocytes the rupture of the germinal vesicle eventually resulted in fragmentation of the oocyte proper without formation of the polar body and the fertilization membrane. In DTT-matured, unfertilized oocytes, artificial rupture of the germinal vesicle before its normal breakdown caused parthenogenetic development following polar body formation. Pipetting after normal germinal vesicle breakdown had no such effects. Pipetting did not cause immediate elevation of the fertilization membrane and pipetted oocytes remained fertilizable. These results indicate that the direct outcome of rupturing the germinal vesicle is not the activation of the oocyte in the usual sense of the term, but a provision of some conditions for the oocyte to initiate cleavage division cycles after the meiotic divisions. It is suggested that the germinal vesicle materials contain some factors that induce the "activation" of the oocyte.

Introduction

In meiotic maturation the germinal vesicle contents are intermingled with the cytoplasm after disappearance of the nuclear membrane. Several investigators have reported that sperms incorporated into oocytes at the germinal vesicle stage form the male pronucleus only after germinal vesicle breakdown (Hirai, 1976; Moriya and Katagiri, 1976; Usui and Yanagimachi, 1976; Elinson, 1977; Longo, 1978; Schuetz and Longo, 1981). Observations of oocyte-fragments deprived of the germinal vesicle indicate that pronucleus formation and subsequent initiation of cleavage after fertilization require the interaction or mixing of germinal vesicle materials with the cytoplasm after its breakdown (Costello, 1940; Katagiri and Moriya, 1976; Skoblina, 1976).

In sea cucumber oocytes meiotic division can be induced and completed by DTT treatment, independently of fertilization (Maruyama, 1980). However, the DTT-matured, unfertilized eggs are arrested at the pronucleus stage and do not initiate development.

To analyze relationships between the nucleo-cytoplasmic interaction and initiation of cleavage division cycles, I attempted to rupture the germinal vesicle in either immature or DTT-matured unfertilized oocytes and investigated the effects of precocious mixing or interaction of the germinal vesicle contents with the cy-

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Abbreviations: DTT, dithiothreitol; PB, polar body.

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toplasm. Precocious mixing or interaction of the germinal vesicle contents with the cytoplasm in DTT-matured, unfertilized oocytes induced initiation of cleavage division cycles after two cycles of meiotic division. These "activated" eggs developed parthenogenetically to auricularia larvae that appeared morphologically normal.

Materials and Methods

Gametes

The sea cucumber *Holothuria leucospilota* was collected near the Seto Marine Biological Laboratory of Kyoto University. Females were isolated from males and cultured in running seawater for 1 day or more. After the body surfaces of the females were washed with fresh water, their gonads were removed, washed twice with artificial seawater (Jamarin U, Jamarin Lab., Osaka), and then minced with fine forceps. Oocytes thus isolated were washed twice with artificial seawater. Oocyte maturation was induced by incubation in 1 m*M* dithiothreitol (DTT) solution for 8–10 min (Maruyama, 1980). Immediately after DTT treatment the oocytes were rinsed two or three times to remove DTT. Oocytes or eggs were cultured in artificial seawater at 27–30°C. In the present study, batches in which 90–99% of oocytes matured by DTT treatment were used. In sperm activation experiments, a sperm suspension was prepared from "dry" sperm prior to insemination.

Rupture of the germinal vesicle (pipetting treatment)

Germinal vesicles of unfertilized oocytes were ruptured by mechanical treatment. About 1 ml of oocyte suspension was aspirated 10–15 times through a pipette with a tip diameter of 700 μm (5 times the oocyte diameter). To avoid wounding the oocyte surface, the minimal amount of pipetting to rupture the germinal vesicle was chosen for each batch of oocytes; and, when damage on the oocyte surface was detected after pipetting, the oocyte suspension was discarded. With this simple method, which will be termed "pipetting", the germinal vesicles of all oocytes, regardless of length of DTT treatment, were disintegrated and then disappeared.

Cinematographic observation

To observe development precisely, the pipetted oocytes, unfertilized or fertilized, were filmed at intervals of 15 sec or 1 min with a Bolex camera equipped with a Nikon CFMA time-lapse instrument. Films were analyzed by a Nac Dynamic Frame Analyzer.

Results

Irreversible changes induced by rupture of the germinal vesicle membrane in immature oocytes (without DTT treatment)

Pipetting ruptured the germinal vesicle membrane in full-grown oocytes (Figs. 1A, 1B), including those with a follicle coat (*cf.* Fig. 2A). Immediate light-microscopic examination revealed that the germinal vesicle usually ruptured in its original position, the center of the oocyte. No damage was found on the oocyte surface. When observed several hours later, the ruptured germinal vesicle was neither reconstructed into its original shape nor became visible. Pipetting did not cause formation of the fertilization membrane and the polar body. About 2 h after pipetting, coaggregation of pinkish pigment granules produced a pink spot near the

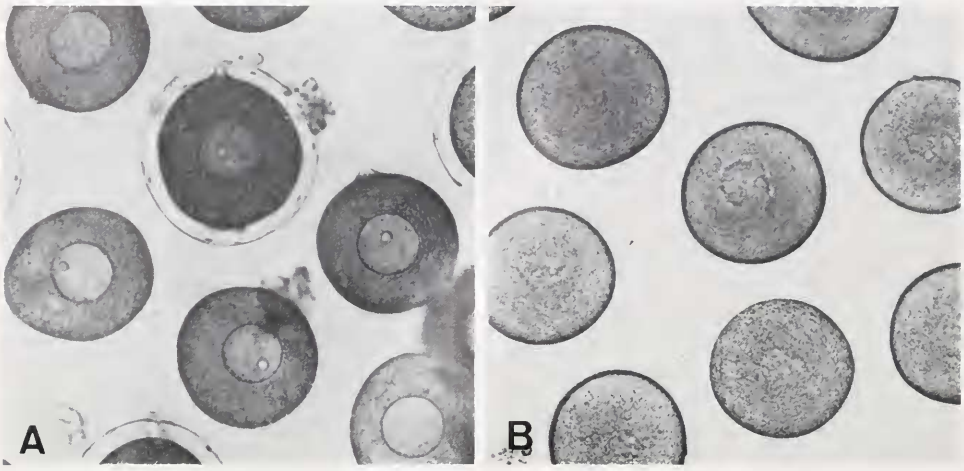

FIGURE 1. Rupture of the germinal vesicle by pipetting. A: intact full-grown oocytes with the germinal vesicle. B: oocytes immediately after pipetting (not induced to mature by DTT).

residue of the germinal vesicle material in some batches of oocytes. Then, all of pipetted oocytes deformed and blebs appeared on the surface. Fragmentation of the oocyte proper followed. By contrast, intact oocytes, with the germinal vesicle, retained their shapes for 20 h or more, even in oocytes that had formed multiple fertilization cones after insemination (Maruyama, 1980).

These results indicate that artificial rupture of the germinal vesicle in immature oocytes induces some irreversible change(s) in physiological activities, leading to fragmentation of the oocyte.

Parthenogenetic development triggered by precocious breakdown of the germinal vesicle in DTT-treated oocytes

Normal events of DTT-induced meiosis in intact, full-grown oocytes are (1) migration of the germinal vesicle to the micropyle process and appearance of a transparent cytoplasmic spot in the subsurface cytoplasm of the antipole of the micropyle process at 15–18 min after the start of DTT treatment at 27–28°C, (2) breakdown of the germinal vesicle at the micropyle process at 20 min, and (3) sequential pinching-off of the first and second polar bodies from the micropyle process (Maruyama, 1980). The unfertilized eggs thus matured were arrested at the pronucleus stage or sometimes at irregular two or four cell stages, and all eggs eventually degenerated through blebbing and fragmentation. In some batches a small percentage (up to 4%) of DTT-matured oocytes developed parthenogenetically.

Artificial rupture of the germinal vesicle by pipetting was also found to be possible at various times after the start of DTT treatment. Pipetting within 20 min after the start of DTT treatment thus produced *precocious* breakdown of the germinal vesicle. The germinal vesicle disintegrated in its original position at the time of pipetting: in the center of the oocyte before 15 min, near the micropyle process between 15 min and 18 min, and at the micropyle process after 18 min. Immediate light-microscopic examination revealed that the oocyte surface was not damaged (see Fig. 2A), although pipetting after 20 min sometimes affected the oocyte sur-

face. The disintegrated germinal vesicle was not reconstructed into its original shape. No elevation of the fertilization membrane was observed, up to 30–50 min after the start of DTT treatment.

A proportion of DTT-treated, unfertilized oocytes whose germinal vesicles had been broken precociously were found to develop parthenogenetically (Fig. 2). Figure 3 shows relationships between the time of pipetting and the frequency of blastulae or gastrulae that were induced to develop parthenogenetically. The efficiency of pipetting in triggering this development was time-dependent. Pipetting at 10 min after the start of DTT treatment gave the maximum efficiency; 20–77% of the pipetted oocytes developed parthenogenetically. Pipetting of intact oocytes at 20 min before DTT treatment was also effective in some batches of oocytes (Fig. 3). However, pipetting at the stages after the normal germinal vesicle breakdown had no effect in triggering the development; these eggs became fragmentated after two cycles of meiotic division.

In the following experiments, pipetting was made routinely at about 10–11 min after the start of DTT treatment.

When maturation of oocytes pipetted at 10–11 min following DTT treatment was observed with time-lapse cinematography, the residue of the germinal vesicle material could be distinguished from the surrounding cytoplasm as a clear area. At 15–20 min after the start of DTT treatment (corresponding to the stage of germinal vesicle migration in control oocytes), the residue of the ruptured germinal vesicle in the pipetted oocytes also migrated to the micropyle process. Almost coincidently, a transparent cytoplasmic spot (termed "clear spot") was formed in

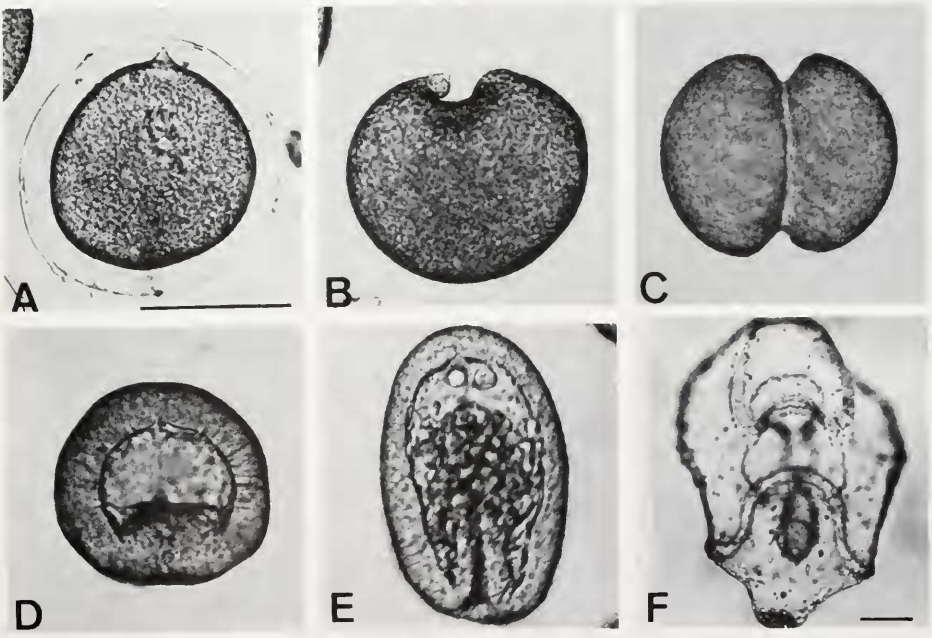

FIGURE 2. Parthenogenetic development of pipetted, DTT-treated oocytes. Oocytes were treated with DTT and then pipetted at 10–11 min after the start of DTT treatment. A: oocyte immediately after pipetting. The follicle coat was stripped off later. B: unilateral deformation (DEF) in an egg forming both polar bodies. C: first cleavage. D: gastrula at 16 h. E: late gastrula with mesenchyme cells at 24 h. F: auricularia at 4.5 days. Bars: 100 μm (the same magnification from A to E).

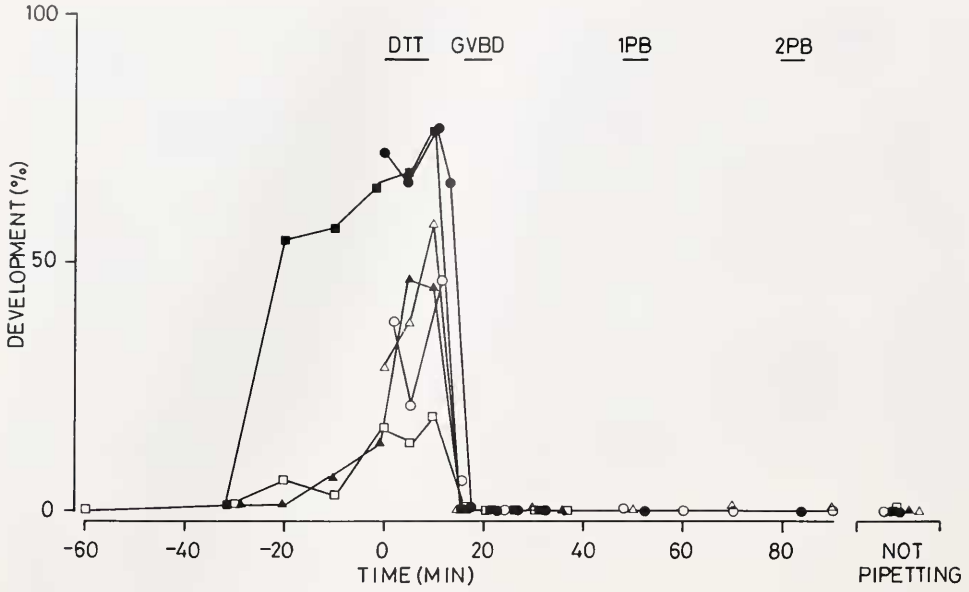

FIGURE 3. Frequencies of parthenogenetic development in unfertilized oocytes pipetted at various times before, during, or after DTT treatment. Abscissa: time (min) of pipetting before or after the start of DTT treatment. Not-pipetting shows controls that were matured by DTT, but not pipetted. Ordinate: percentage of pipetted oocytes developing to blastulae or gastrulae by 11–18 h; DTT, duration of DTT treatment (about 9 min); GVBD, germinal vesicle breakdown in not-pipetted controls; 1PB, first polar body; 2PB, second polar body. Results in 6 batches of oocytes are shown. Each point represents about 100 oocytes. Temperature was 29–30°C.

the subsurface cytoplasm of the antipole of the micropyle process. Then, from 30 min to 50 min, a considerable proportion of the pipetted, unfertilized, and DTT-treated oocytes were observed to elevate the fertilization membrane "spontaneously." This elevation of the fertilization membrane followed transient surface wrinkling, just as in inseminated, DTT-matured controls. The fertilization membrane subsequently sank and adhered to the egg surface.

Table I shows polar body formation in pipetted, unfertilized, and DTT-treated oocytes. In 68% of the oocytes, the first and second polar bodies were formed from the micropyle process, nearly at the same time as in DTT-matured controls. In 24% of the oocytes, no formation of the second polar body was observed. In 8% of the oocytes, both polar bodies were not formed. In this way, most of the oocytes (92%) formed polar bodies. Most oocytes forming one or two polar bodies began parthenogenesis as shown in Table I and Figure 4. These results indicate that failure of polar body formation itself does not causally relate to initiation of cleavage division cycles in these pipetted oocytes.

Figure 4A shows timetables of parthenogenesis in the pipetted, unfertilized, and DTT-treated oocytes (shown in Table I) that formed two polar bodies (2PB), one polar body (1PB), or no polar bodies (0PB).

2PB eggs. An event specific to the eggs was transient formation of a unilateral deformation (DEF) (Fig. 2B), with accumulation of pinkish pigment granules in the deformed region. This appeared at about 130 min and regressed at 150 min when the eggs resumed a spherical shape with dispersal of the accumulated pigments. The deformed region appeared random as to the position of polar bodies

TABLE I

Polar body formation in pipetted, unfertilized, and DTT-treated oocytes

Batch	No. of oocytes observed		
	2PB	1PB	0PB
A	15 (10)	5 (0)	0 (0)
B	9 (6)	7 (4)	5 (5)
C	19 (15)	3 (3)	0 (0)
Totals	43 (31)	15 (7)	5 (5)
% of totals	68 (72)	24 (16)	8 (12)

2PB, 1PB, and 0PB, respectively, indicate oocytes forming the first and second polar bodies, those forming one polar body, and those forming no polar body. These oocytes were continously observed from 20 min to 210 min after the start of DTT treatment at 29–30°C. Numerals in parentheses show the number of oocytes which began parthenogenesis in the population of oocytes observed. Pipetting was made at 10–11 min.

on the oocyte surface. About 30–35 min after appearance of the deformation, first cleavage (Fig. 2C) occurred as in inseminated control eggs. At that time, a similar deformation appeared again in a small percentage (about 6%) of these eggs. Subsequent cleavages took place at intervals of 30–35 min.

1PB eggs. Eggs failed to form the second polar body, and surface deformation near the micropyle process was observed nearly at the same time as second polar body formation in the controls. At about 135 min, first cleavage took place as in the inseminated control eggs. Subsequent cleavages occurred at intervals of 30–35

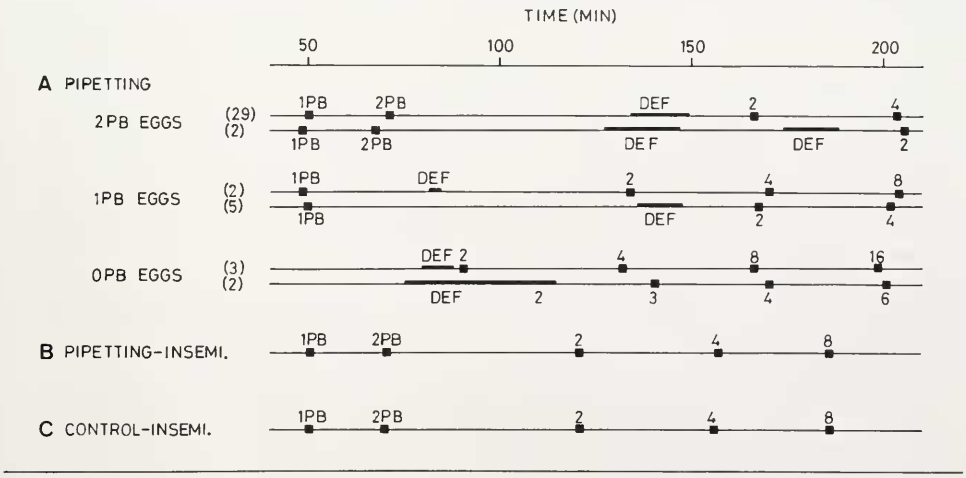

FIGURE 4. Timetables of developmental events in pipetted, unfertilized oocytes (A); pipetted, inseminated oocytes (B); control, inseminated oocytes (C). In each event the mean of the initiation time was indicated by the closed box. All oocytes were treated by DTT for 9–10 min. Pipetting was at 10–11 min for A and B, and insemination was at 23 min for B and C. Abscissa: time (min) after the start of DTT treatment. 1PB, first polar body; 2PB, second polar body; DEF, unilateral deformation; Arabic number, stages expressed by cell number. Numerals in parentheses in A indicate the number of oocytes observed; these were the identical oocytes as shown in parentheses in Table I. Temperature was 29–30°C.

min. In most of the eggs (5 out of 7), such a unilateral deformation as observed in 2PB eggs took place at 135–150 min, and first cleavages in these occurred 30–35 min later.

0PB eggs. The first and second polar bodies were not formed. Immediately after second polar body formation in the inseminated control eggs, a unilateral furrow-like constriction appeared, and then the eggs divided into two cells with one nucleus in each cell. Most of them became four cells at about 130 min. In some only one of the two cells divided producing a three cell stage. Subsequent cleavages occurred at intervals of 30–35 min. This group of eggs was considered at the most advanced "stages" in the parthenogenetically developing egg population.

As shown in Figure 4, timing of each cleavage or deformation was similar, except for the two cell stage in 0PB eggs, which was delayed 10–20 min as compared to control inseminated eggs. Their intervals between cleavages (deformation) were similar to those in control inseminated eggs. Most of these cleaving eggs developed to gastrulae. The gastrulae began to move by ciliary motion and hatched from the fertilization membrane.

Further development in the pipetted, unfertilized, and DTT-treated eggs was similar to that in normally fertilized eggs. Figures 2D and 2E, respectively, show free swimming early and late gastrulae. They swam with the animal pole leading, accompanied with counter-clockwise spin as in normally developing embryos (Maruyama, 1980). Later, the dorsal pore and pore canal formed; the dorsal pore shifted to the left side from the middle line of the body, seen from the dorsal side of the body. By 3 days, they developed to typical auricularia larvae (Fig. 2F). A birefringent body formed at the posterior end of the body and the ciliary ridge clearly formed a single loop.

Fertilizability and post-fertilization development in DTT-treated, pipetted oocytes

DTT-treated oocytes with germinal vesicles ruptured precociously by pipetting at 10–11 min were inseminated at various times after the start of DTT treatment. Insemination from 23 min to 40 min caused immediate surface wrinkling, formation of the fertilization cone, and elevation of the fertilization membrane in all of the oocytes. Most of these oocytes (70–100%) had a single fertilization cone. However, insemination after 50 min did not cause surface wrinkling and formation of the fertilization cone. Most of the pipetted, uninseminated, and DTT-treated oocytes elevated fertilization membranes at 50 min. By contrast, at these times, not-pipetted, DTT-matured oocytes were fertilized as evidenced by surface wrinkling, fertilization cone, and elevation of the fertilization membrane upon insemination. In this way, the pipetted, DTT-treated oocytes were fertilized when inseminated before their "spontaneous" elevation of the fertilization membrane.

Figure 5 shows timing of first cleavage in DTT-treated oocytes that were pipetted at 10–11 min and inseminated at 23 min after the start of DTT treatment. First cleavages occurred at the same time as in the control DTT-matured oocytes that had been inseminated at 23 min. The subsequent cleavages took place at the same time as in control inseminated oocytes (Figs. 4B, 4C). These pipetted, inseminated oocytes showed no pre-cleavage deformation. Most of them (80%) developed to gastrulae by 14 h. This contrasts with the pipetted, uninseminated oocytes, in which only 50% (Fig. 5) developed with some delay, after pre-cleavage deformation.

These results indicate that DTT-treated, pipetted oocytes can be fertilized by

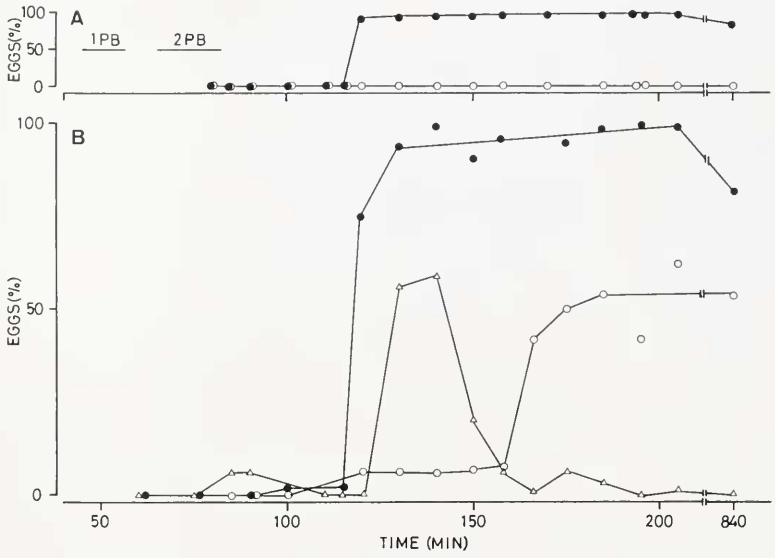

FIGURE 5. Effects of sperm activation in DTT-treated, pipetted oocytes and control oocytes. Abscissa: time (min) after the start of DTT treatment. Ordinate: percentage of cleaving eggs. A: control oocytes which were inseminated at 23 min (closed circles), or not inseminated (open circles), following DTT treatment for 9 min. B: oocytes that were pipetted at 10–11 min following DTT treatment and were either inseminated at 23 min (closed circles) or not inseminated (open circles). The open triangles show the oocytes that exhibit unilateral deformation among the pipetted, unfertilized oocytes. The oocytes derived from the same batch were used for A and B. Each point represents about 100 oocytes.

sperm, and that fertilization switches the pattern of development to that in normally fertilized oocytes.

DISCUSSION

The present study shows that (1) the germinal vesicle in sea cucumber oocytes can be ruptured by pipetting, (2) oocytes with ruptured germinal vesicles change their physiological activities irreversibly, (3) the pipetted, unfertilized oocytes can be induced to mature by DTT treatment, and, (4) when the germinal vesicle is ruptured before its normal breakdown, most of the DTT-treated oocytes begin parthenogenetic development after meiosis.

This pipetting method differs in an essential feature from previously reported methods of inducing parthenogenesis in echinoderm eggs (Lillie, 1915; Harvey, 1956; Epel, 1979). In these methods the effective period of activation treatment coincides with a period during which eggs can be triggered to develop by normal fertilization. Responses of the eggs following the artificial activation, therefore, are similar, in their properties and timing, to those in eggs activated by fertilization. In the pipetting method of parthenogenesis, the effective time for the treatment was before the normal fertilizable time, or before breakdown of the germinal vesicle (see Maruyama, 1980).

The primary target of the pipetting treatment may be the egg surface. However, due to the thick jelly coat and follicle coat surrounding the oocyte, aspiration of the oocytes through the pipette did not seem to affect the integrity of the oocyte surface. The only change observed was rupture of the germinal vesicle membrane.

Capability of monospermic fertilization, as evidenced by the single fertilization cone and elevation of the fertilization membrane in DTT-treated, pipetted oocytes upon insemination, may also indicate that pipetting itself does not directly cause a cortical reaction. On the other hand, the time-dependence of efficiency of pipetting for inducing parthenogenesis indicates that a meiotic phase is important in triggering parthenogenetic development. Precocious "breakdown" of the germinal vesicle may be the principal cause of parthenogenetic development. It may be concluded that its resultant precocious interaction (or mixing) of the germinal vesicle contents with the cytoplasm causes initiation of cleavage division cycles.

DTT-treated, unfertilized oocytes were arrested at the pronucleus stage or sometimes at irregular two or four cell stages. Naturally spawned oocytes were also arrested at the pronucleus stage after two cycles of meiotic division when they were not inseminated, and all of them eventually fragmentated (Maruyama, unpublished). These observations may suggest presence of some mechanisms that inhibit initiation of cleavage division cycles after meiosis and can be normally triggered by fertilization, as the mechanism of metaphase II arrest in amphibian oocytes (Masui and Clarke, 1979). Unfertilized oocytes whose germinal vesicles had been broken either by DTT maturation in oocytes not-pipetted, by natural maturation, or by pipetting in immature oocytes were not arrested at some stable states but became fragmentated eventually. These observations and "spontaneous" elevation of the fertilization membrane accompanying surface wrinkling in pipetted, unfertilized, and DTT-treated oocytes suggest that the germinal vesicle materials contain some factors that activate physiological occurrences in the oocyte. When this activation occurs at a suitable time during the maturation process induced by DTT treatment, the oocytes may begin parthenogenetic development. As shown by fertilizability of pipetted, DTT-treated oocytes, however, this "activation" does not directly activate the oocyte in the usual sense of the term, but is a provision of some condition for the oocytes to initiate parthenogenesis.

First cleavage in pipetting-induced parthenogenesis occurred considerably later than in normally fertilized eggs and its timing varied among types of eggs as to the number of polar bodies formed (Fig. 4). However, cleavages or deformation occurred at the same time among 2PB, 1PB, and 0PB eggs (Fig. 4). Such coincidence of their timing suggests that these eggs were determined to initiate parthenogenetic development at an identical point in time.

Time-dependence of pipetting to induce parthenogenesis in DTT-treated oocytes, and irreversible changes of physiological activities in immature oocytes with ruptured germinal vesicles indicate that untimely disappearance of the nuclear membrane during cell cycle may affect normal expression of cytoplasmic activities. Thus, the nuclear membrane probably functions as a barrier against untimely exposure of the karyoplasm (or chromosomes) to its cytoplasm.

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